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Treatment with Tertiary Oximes Prevents Seizures and Improves Survival Following Sarin Intoxication

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Abstract The capability of the tertiary oximes, monoisonitrosoacetone (MINA) and diacetylmonoxime (DAM), to reactivate acetylcholinesterase (AChE) inhibited by sarin (GB) in the blood, brain, and peripheral tissues of guinea pigs was compared with that of the quaternary oximes 2-PAM, HLö7, and MMB-4. Animals were injected subcutaneously (s.c.) with $1.0\times LD_{50}$ of GB and treated intramuscularly (i.m.) 5 min later with one of these oximes. Sixty minutes after GB exposure, tissues were collected for AChE analysis. At low doses, MINA and DAM produced significant increases in AChE activity in all brain areas examined, but no significant AChE reactivation in peripheral tissues or blood. At higher doses, MINA and DAM increased AChE activity in the brain, peripheral tissues, and blood. In contrast, the quaternary oximes produced significant reactivation in peripheral tissues and blood AChE, but no significant reactivation of brain AChE. In another study, animals were pretreated i.m. with pyridostigmine 30 min prior to s.c. challenge with $2.0\times LD_{50}$ of GB and treated i.m. 1 min later with atropine sulfate (2.0 mg/kg), plus a varied dose of oximes. MINA and DAM prevented or terminated GB-induced seizure activity and protected against GB lethality in a dose-dependent fashion. In contrast, none of the quaternary oximes prevented or stopped GB-induced seizures. Thus, tertiary oximes reactivated AChE in the brain, improved survival, and terminated seizures following GB intoxication.

Keywords Sarin · Acetylcholinesterase · Oxime reactivator · Tertiary oxime · Anticonvulsant · Seizures

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Introduction

Organophosphorus (OP) nerve agents, such as sarin (GB), are extremely potent inhibitors of the cholinesterase (ChE) enzymes. Their toxic effects are due to hyperactivity of the cholinergic system as a result of the inhibition of ChE, in particular, acetylcholinesterase (AChE), and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (Karczmar 1967, 1970; Taylor 2001). Exposure causes a progression of toxic signs, including hypersecretions, muscle fasciculations, tremor, convulsions, respiratory distress, and death (Karczmar 1967, 1970; Taylor 2001). A combined regimen of prophylaxis and therapy is the recommended medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (Dunn and Sidell 1989). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine bromide (PB), shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents (Dirnhuber et al. 1979). In the event of nerve agent poisoning, immediate therapeutic treatment with an anti-

cholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites, while an oxime, such as pyridine-2-aldoxime methyl chloride (2-PAM), is used to reactivate any unaged, inhibited ChE (Wilson and Ginsburg 1955; Taylor 2001).

2-PAM is the oxime currently used in the U.S. for the emergency treatment of nerve agent exposure. Other countries use bispyridinium compounds such as obidoxime, TMB-4, or HI-6 as oxime antidotes (Moore et al. 1995). These oximes are positively charged quaternary nitrogen structures, which renders them unable to cross the blood–brain barrier (BBB). The inability of quaternary oximes to enter the brain and reactivate brain AChE is a major limitation of current oxime therapy, since the brain is a major target of OP nerve agents (Taylor 2001).

Monoisonitrosoacetone (MINA) and diacetylmonoxime (DAM) are two tertiary oximes that were investigated in the 1950s. When used alone or in combination with atropine sulfate, MINA and DAM were shown to raise the LD_{50} doses of GB in several animal species (Askew 1957; Dultz et al. 1957; Rutland 1958; Myers 1959). Since both are highly lipid-soluble and readily penetrate the BBB (Cohen and Wiersinga 1960), they are expected to reactivate AChE within the central nervous system (CNS). Unfortunately, studies of these two tertiary oximes were not pursued further due to reports that quaternary pyridinium oximes (e.g., 2-PAM) were more potent reactivators of phosphorylated AChE by several orders of magnitude in human erythrocytes (Hobbiger 1963).

The two studies reported here were performed to compare the tertiary oximes MINA and DAM with several quaternary oximes, 2-PAM, MMB-4, and HLö7, first, for their ability to reactivate GB-inhibited AChE activity in the blood, peripheral tissues, and brain, and secondly, for their ability to control GB-induced seizures and improve survival.

Materials and Methods

Subjects

Male Hartley guinea pigs (Crl:(HA) BR COBS) (250–300 g) were purchased from Charles River Labs (Kingston, NY, USA). They were individually housed in temperature-controlled (21±2°C) and humidity-controlled (50±10%) quarters maintained on a 12-h light–dark schedule (lights on at 0600). Laboratory chow and water were freely available when the animals were in home cages.

Materials

Atropine sulfate, PB, MINA, DAM, 2-PAM, HLö7, and MMB-4 were purchased from commercial sources. They were prepared in saline for intramuscular (i.m.) injection. GB was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD, USA). It was diluted in ice-cold saline prior to subcutaneous (s.c.) injection. All injection volumes were 0.5 ml/kg.

Reactivation Experiment

Prior to the experiment, ~0.5 ml blood was drawn (Vallejo-Freire 1951) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBC). On the day of the study, groups of guinea pigs were injected s.c. with either saline (0.5 ml/kg) or a 1.0× LD_{50} dose of GB (42.0 μg/kg). Five minutes later, when the inhibition of AChE activity by GB reached maximum (Shih et al. 2005), saline (0.5 ml/kg), HLö7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25.0 mg/kg), MINA (12.63 mg/kg), or DAM (14.66 mg/kg) was given i.m. Control animals received s.c. saline (no GB) and i.m. saline (no oximes). Sixty minutes after saline or GB administration, the animals were deeply anesthetized with isoflurane and euthanized by decapitation. Shortly before anesthesia, the severity of toxic signs of each animal was scored (see below). Blood (RBC and WB), brain (brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum), and peripheral tissues (diaphragm, heart, and skeletal muscle) were collected. Samples were processed for AChE activity and protein concentrations according to the methods described by Shih et al. (2005). Later, dose–response effects of MINA and DAM on AChE activity were also investigated.

Treatment Dose Rationale

The 25-mg/kg dose of 2-PAM approximates the total dose of 2-PAM in three autoinjectors (600 mg per injector) given as immediate nerve agent treatment to a 70- to 75-kg human. The 25-mg/kg dose of 2-PAM is equivalent to a 145-μmol/kg dose, and the initial MINA and DAM doses (12.63 and 14.66 mg/kg, respectively) were matched to this. Later, additional doses of MINA (17.5, 35, 60, or 80 mg/kg, i.m.) and DAM (23, 41, 73, or 128.8 mg/kg, i.m.) were also examined. MMB-4 and HLö-7 are bispyridinium compounds similar to HI-6; a 58-μmol/kg dose was used for MMB-4 and HLö7, based on a suggested three-autoinjector equivalent dose (1,500 mg) of HI-6 (Clair et al. 2000).

Toxic Signs Test

At 58 min after GB injection, guinea pigs were scored for signs of nerve agent intoxication using a score system modified from one reported for rats (Shih and Romano 1988). Animals were scored for absence [0] or presence [1] of each of the following signs: salivation, lacrimation, and

nystagmus. General motor signs were assessed using a 0–3 score: normal=0, fasciculation=1, tremor=2, or convulsion =3. Next, the animal was allowed to walk on the bench top and general state was assessed using a 0–3 score: normal= 0, mild uncoordination=1, impaired movement/with righting reflex=2, or prostration/no righting reflex=3. A cumulative score was calculated for each subject (maximal score=9). A cumulative score was categorized as mild [1–3], moderate [4–6] or severe intoxication [7–9].

Anticonvulsant Experiment

Animals were implanted 1 week prior to experimentation with stainless-steel cortical electrodes to record electroencephalographic (EEG) signals according to the methods described earlier (Shih et al. 2007). On the day of study, after a 15-min recording of baseline EEG activity, animals received a dose of PB (0.026 mg/kg, i.m.) to produce a 20–30% blood AChE inhibition (Lennox et al. 1985). Thirty minutes later, the animals were challenged with $2\times LD_{50}$ (s.c.) GB and 1 min later treated i.m. with atropine sulfate (2.0 mg/kg) plus HLö7 (30.2 mg/kg), MMB-4 (26.0 mg/kg), 2-PAM (25 mg/kg), MINA (26–60 mg/kg), or DAM (82–231 mg/kg). There were five to 12 animals per dose group. Animals were observed continuously for at least 6 h and again for another 30 min at 24 h after exposure. Seizure onset was operationally defined as the appearance of ≥10 s of rhythmic high amplitude spikes or sharp-wave activity in the EEG tracing. Each animal was rated as never having a seizure or having the seizure terminated or not terminated based on the overall appearance of the EEG record at the end of the experimental day and during the 24-h observation. (Note: An animal was rated as terminated if a seizure never occurred or if the seizure terminated and the EEG remained normal at all subsequent observation times.) Animals rated as not terminated had obvious epileptiform activity that never stopped in response to treatment or reappeared in the EEG record before the end of the 6-h recording on the day of exposure and/or during the 30-min recording at the 24-h observation time. Mortality and body weight of survivors were recorded 24 h after GB exposure. Body weight changes are an indicator of long-term health and survival following nerve agent exposure (Shih et al. 1990; McDonough et al. 1998).

Data Analysis

AChE activity was expressed as micromoles per milliliter per minute for blood and as micromoles per gram of protein per minute for brain and tissue. The enzymatic activities of

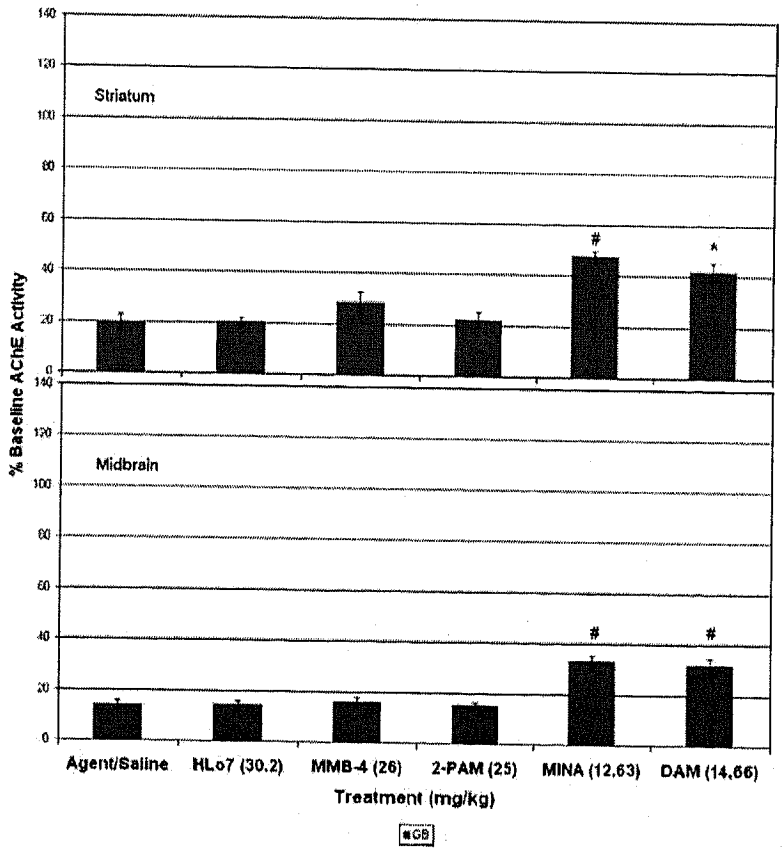

Figure 1 AChE activity in the striatum and midbrain regions of the guinea pigs exposed to the agent GB ($1.0\times LD_{50}$, s.c.) and treated i.m. 5 min later with saline (as control), 58 μmol/kg of HLö7 and MMB-4, or 145 μmol/kg of 2-PAM, MINA, and DAM. Brain tissues were collected at 60 min after GB exposure. AChE activities were expressed as the mean percentage of baseline activity ± SEM with $N=8$ in each treatment group. #$p<0.05$ compared with agent/saline, HLo7, MMB-4, and 2-PAM groups; *$p<0.05$ compared with agent/saline, HLo7, and 2-PAM groups

the treatment groups were then calculated as the percentage of the saline–saline control group. Statistical analyses of enzymatic activities and body weight change were performed using one-way analysis of variance to compare across treatments. A post hoc Tukey test was used for multiple comparisons. A Dunnett *C* post hoc test was used in cases where equal variances could not be assumed. Differences in incidence of toxic signs between treatment groups were evaluated using Fisher's exact test. Statistical significance was defined as $p<0.05$. A probit regression analysis (SPSS for Windows, Version 14.0, Chicago, IL, USA) was used to estimate the anticonvulsant ED_{50} values along with the 95% confidence intervals for each oxime treatment.

Results and Discussion

Reactivation Experiment

Signs of Toxicity and Lethality

All guinea pigs exposed to a $1.0\times LD_{50}$ of GB and not receiving any therapy showed a high incidence (93%; 14 of 15 animals) of toxic signs rated in the moderate range (average score=4.07) and three animals died within 60 min. Animals treated with 2-PAM, MMB-4, or HLö7 all showed a similar incidence (100%) of toxic signs as did the saline controls and were rated in the mild to moderate range (average scores=2.75 to 4.13). The number of animals showing signs of nerve agent intoxication among those treated with MINA (26%, ten of 39) or DAM (20%, eight of 40; $p<0.001$; Fisher's exact test) was significantly smaller than that among the saline-treated controls, and the severity of signs was mild (range from 0.00 to 0.88). No animal treated with any quaternary or tertiary oxime died within 60 min of GB exposure.

AChE Activity in Brain Regions

Sixty minutes following exposure to a $1.0\times LD_{50}$ dose of GB, AChE activities in the brainstem, cerebellum, cortex, hippocampus, midbrain, spinal cord, and striatum were inhibited to 19%, 11%, 10%, 24%, 14%, 30%, and 20% of control, respectively. Figures 1 and 2 show the typical AChE reactivation effects observed in two brain regions, the striatum and midbrain. As can be expected, none of the quaternary oximes (2-PAM at 145 μmol/kg; MMB-4 and HLö7 at 58 μmol/kg) showed any AChE reactivation in the CNS, since they do not penetrate the BBB due to their quaternary structure and limited lipid solubility. On the

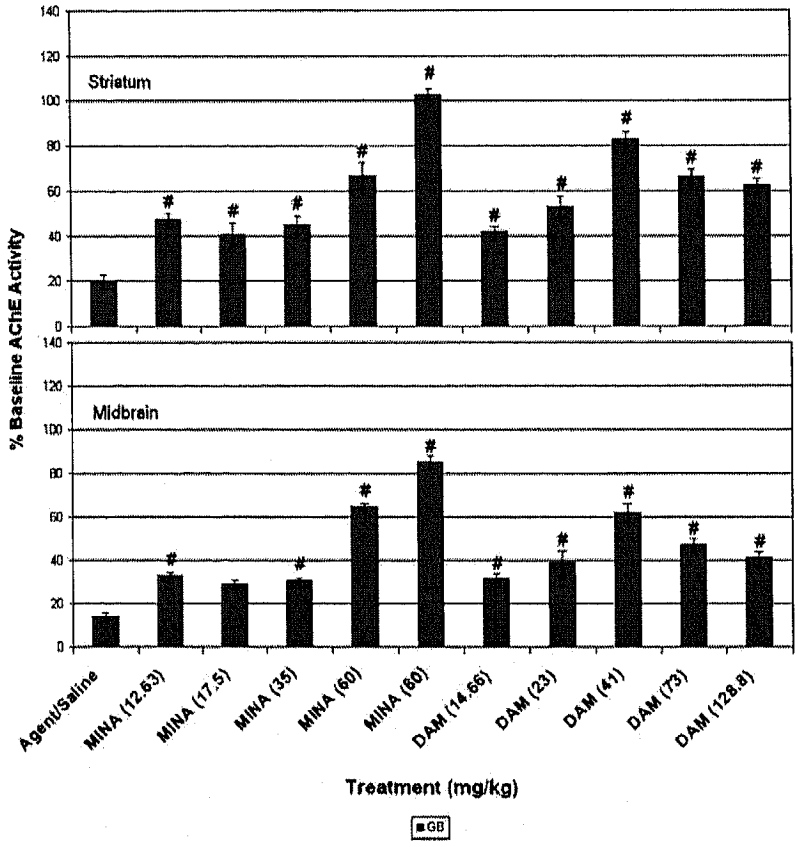

Figure 2 AChE activity in the striatum and midbrain regions of the guinea pigs exposed to the agent GB ($1.0\times LD_{50}$, s.c.) and treated i.m. 5 min later with saline (as control) or various doses of MINA or DAM. Brain tissues were collected at 60 min after GB exposure. AChE activities were expressed as the mean percentage of baseline activity ± SEM with $N=8$ in each treatment group. #$p<0.05$ compared with agent/saline (control) group

Table 1 Effects of oxime on GB-induced seizure occurrence, termination, and survival

Guinea pigs were pretreated with pyridostigmine bromide (0.026 mg/kg, i.m.) 30 min prior to GB ($2\times LD_{50}$, s.c.) and 1 min later by atropine sulfate (2.0 mg/kg, i.m.) and a dose of an oxime (i.m.). EEG seizure onset, termination, and 24-h survival were recorded (number responded/total numbers of animals). Numbers of animals responded in the Seizure "off" column included both animals that never seized and animals that seized initially but seizure activity spontaneously terminated soon after

Treatment (mg/kg)	Never seized	Seizure "off"	Survival (24h)
2-PAM (25)	0/10 (0%)	0/10 (0%)	5/10 (50%)
MMB-4 (26)	0/5 (0%)	0/5 (0%)	2/5 (40%)
HLö7 (30.2)	0/5 (0%)	0/5 (0%)	1/5 (20%)
MINA			
(20)	0/7 (0%)	0/7 (0%)	3/7 (43%)
(26)	1/11 (9%)	1/11 (9%)	7/11 (64%)
(35)	2/12 (17%)	7/12 (58%)	9/12 (75%)
(46)	6/10 (60%)	9/10 (90%)	9/10 (90%)
(60)	9/12 (75%)	11/12 (92%)	12/12 (100%)
DAM			
(41)	0/7 (0%)	0/7 (0%)	5/7 (71%)
(73)	1/6 (17%)	1/6 (17%)	5/6 (83%)
(129)	4/6 (67%)	4/6 (67%)	6/6 (100%)
(231)	6/6 (100%)	6/6 (100%)	6/6 (100%)

other hand, MINA and DAM are tertiary structures and highly lipid-soluble. At a dose of 145 μmol/kg, MINA reactivated AChE activity significantly in the brainstem, midbrain, and striatum, while DAM significantly reactivated the AChE activity in the midbrain, striatum, spinal cord, and cerebellum. The differences in the regional specificity of these two tertiary oximes are not understood, but may be due to their individual distribution profile in different brain regions (Shih et al. 2005). However, at higher doses the AChE, reactivating capacity in the CNS was highly significant for both MINA and DAM, with MINA being more potent than DAM. MINA produced a dose-dependent (12.83–80 mg/kg) reactivation of brain AChE activity. DAM, on the other hand, produced an inverted U-shaped curve. Doses from 14.66 to 41 mg/kg DAM produced increasing levels of AChE activity that reached a plateau at 41 mg/kg, then with higher doses of DAM (73 and 128.8 mg/kg), there was a trend of reducing AChE activity, although these were still significantly higher than in GB-exposed saline-treated control animals. The reason for this effect of DAM is not clear, but is probably due to DAM binding initially to carboxylesterase in the plasma of guinea pigs after absorption and reactivating OP-inhibited carboxylesterase, thus reducing its availability to bind and reactivate AChE (Myers 1959).

AChE Activity in Peripheral Tissues and Blood

Sixty minutes following exposure to a $1.0\times LD_{50}$ dose of GB, AChE activities in the diaphragm, heart, and skeletal muscle were inhibited to about 28%, 17%, and 40% of control, respectively, and in RBC and WB to about 7% and 9% of control, respectively. In the peripheral tissues and blood, neither MINA nor DAM produced any significant reactivation of GB-inhibited AChE at the low test dose (145 μmol/kg), but did produce significant increases in AChE activity in these tissues at higher doses (60 or 80 mg/kg MINA; 41 mg/kg DAM). In contrast, all three quaternary oximes were readily able to significantly reactivate GB-inhibited AChE in the peripheral tissues and blood. In the diaphragm and skeletal muscle, HLö7, MMB-4, and 2-PAM markedly reactivated GB-inhibited AChE, with HLö7 and MMB-4 both producing significantly greater AChE reactivation than did 2-PAM. In the heart, HLö7, MMB-4, and 2-PAM significantly reactivated AChE activity to a similar degree when compared with the GB-exposed controls. In the RBC, MMB-4 reactivated significantly more AChE activity inhibited by GB than did HLö7 and 2-PAM, while HLö7, MMB-4, and 2-PAM significantly reactivated AChE activity in WB to a similar degree when compared with the GB-exposed controls.

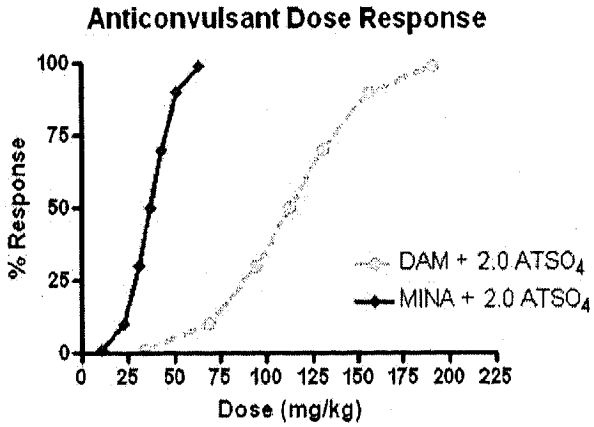

Figure 3 The dose–response curves for the prevention/termination of seizures by MINA and DAM following GB ($2\times LD_{50}$, s.c.) challenge in guinea pigs. All animals were pretreated with pyridostigmine bromide (0.026 mg/kg, i.m.) 30 min prior to GB and followed 1 min later by atropine sulfate (2.0 mg/kg, i.m.) and a dose of an oxime (i.m.)

Under the conditions of this study, both quaternary and tertiary oximes were able to reactivate GB-inhibited AChE, but with notable variations with respect to tissue specificity. Only the tertiary oximes reactivated GB-inhibited AChE in the CNS, whereas quaternary oximes and the higher doses of MINA and DAM readily reactivated GB-inhibited AChE in the blood and peripheral tissues. Additionally, animals treated with MINA and DAM clearly displayed fewer signs of GB-induced cholinergic toxicity than did those animals that were treated with 2-PAM, MMB-4, or HLö7. These observations were also confirmed by the results of the anticonvulsant study.

Anticonvulsant Experiment

Seizure Occurrence and Survival at 24 h

Table 1 shows the incidence of EEG seizure occurrence and 24-h survival in guinea pigs exposed to $2.0 \times LD_{50}$ GB and treated 1 min later with atropine sulfate (2.0 mg/kg, i.m.) plus an oxime. All animals treated with a quaternary oxime (2-PAM, MMB-4, or HLö7) developed continuous seizure activity and only 20–50% of the animals survived 24 h. With MINA at doses of 20, 26, 35, 46, and 60 mg/kg, 0%, 9%, 17%, 60%, and 75% of animals, respectively, never exhibited EEG seizure activity and 43%, 64%, 75%, 90%, and 100% of these animals, respectively, survived 24 h. Similarly, with DAM at doses of 41, 73, 129, and 231 mg/kg, 0%, 17%, 67%, and 100%, respectively, of the animals never exhibited EEG seizure activity and 71%, 83%, 100%, and 100% of these animals, respectively, survived 24 h. Thus, MINA and DAM protected animals from GB-induced seizures and lethality in a dose-dependent fashion, with MINA having greater potency than DAM.

In animals treated with 2-PAM, MMB-4, or HLö7, the EEG seizure activity induced by GB never abated, although, at 24 h, the amplitude and frequency of spiking activity were significantly reduced. Animals treated with DAM either developed seizures or did not. However, some animals treated with MINA (35 mg/kg, N=2; 46 mg/kg, N=3; 60 mg/kg, N=2) developed periods of epileptiform activity that lasted several minutes and then spontaneously terminated; the average termination time was 5.2 min. Figure 3 shows the anticonvulsant dose–response effects of MINA and DAM in the guinea pig model. The anticonvulsant ED_{50} (with 50% confidence limits) for MINA was 36.65 mg/kg, i.m. (0.00–76.30 mg/kg, i.m.), whereas the anticonvulsant ED_{50} for DAM was 112.51 mg/kg, i.m. (83.78–178.87 mg/kg, i.m.).

Animals treated with 2-PAM, MMB-4, or HLö7 experienced a significant weight loss (range=45–58 g) that was equivalent to that shown by the GB-exposed saline-treated controls. In contrast, animals treated with MINA or DAM in which seizures were prevented or spontaneously terminated experienced significantly less ($p<0.001$) body weight loss (range=7–15 g) over the 24-h survival period than did MINA-treated or DAM-treated animals that experienced continuous seizures (range=36–54 g) or the animals treated with 2-PAM, MMB-4, or HLö7. Thus, increasing the doses of MINA or DAM reduced seizure occurrence, increased the propensity for seizure termination, enhanced survival, and minimized overnight weight loss. On the other hand, the quaternary oximes, 2-PAM, MMB-4, and HLö7, had no effect on GB-induced seizure activity and did not protect against GB-induced body weight loss.

In conclusion, this study clearly shows that the tertiary oximes DAM and MINA reactivated AChE in the brain, reduced toxic signs, improved survival, and terminated seizures following GB intoxication. The current results support the notion that central AChE reactivation or preservation of CNS AChE activity following nerve agent intoxication is critical in the medical management of nerve agent intoxication (Wetherell et al. 2002). Thus, tertiary oximes could be an excellent adjunct to current pretreatment and therapy regimens for medical management of nerve agent poisoning.

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